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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: **David Lewis, Hans Herweijer, James E. Hagstrom, Aaron Loomis, Jon A. Wolff,**

Serial No.: **10/007,448** Examiner: **Terra C. Gibbs**

Filed: **11/07/2001**

Group Art Unit: **1635**

For: **Inhibition Of Gene Expression By Delivery Of Small Interfering RNA To Post-Embryonic Animal Cells *In vivo***

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. 1.131

Dear Sir:

I, David L. Lewis, hereby declare as follows:

1. I am an inventor of the processes found in the captioned patent application.
2. Photocopies of pages from my, David Lewis', personal laboratory notebook show experimental evidence of delivery of short polynucleotides to animal cells.
3. It is known to me that the process performed in the notebook pages results in the delivery of expression inhibiting polynucleotides to animal cells as described in my specification.
4. The process was conceived and performed prior to the effective date of the Office Action prior art references indicating delivery of expression inhibiting polynucleotides.
5. Development of this process occurred with due diligence from the date of conception to the filing of the application.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

7/30/04

David L. Lewis, Ph.D. Date

Project No.

Book No. 180 TITLE hydrodynamic tail vein injection of pMIR48 and siRNA

From Page No.

Purpose: Determine if co-injection of DL57:DL58 siRNA with pMIR48 inhibits Luc+ expression

Materials:

- pMIR48
- DL57·DL58 siRNA 40 mM 0.53 mg/L 7/9/01

Methods:

- Group 1 pMIR48 (10 mg) + 0.5 mg DL57:DL58 2 animals each
- Group 2 " + 5 mg DL57:DL58 "
- Group 5 " + 0 mg "

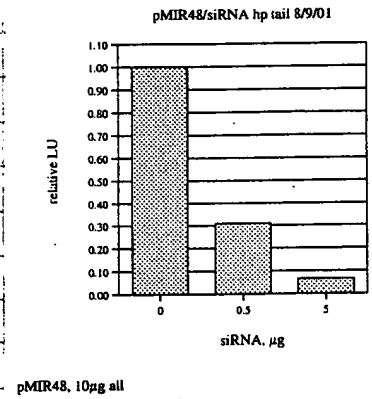
Inject by high pressure tail vein injection in ICR mice
Homogenize liver measure Luc+ activity on Day 1

Results:

Luciferase Assay - Liver tissue

hp tail vein
liver homogenate 20hr
10μg pMIR48 all
assay 10μl normal Luc+ assay

DL57:DL58 siRNA (μg)		LU		AVE	undiluted LU	normalized
0	undil	21286848	33804016			
	1:10	4139650	9485125	6812388	68123875	1
0.5	undil	15414087	12688318			
	1:10	2183854	2030952	2107403	21074030	0.3093487
5	undil	3337360	4820293	4078827	4449560	0.0653157
	1:10	ND	ND			



MEASUREMENT ROUTINE

10 AUG 01 10:03 V.2.03

PROTOCOL NO. : 8 NAME :	10
VOLUME INJ. 1 [ul]	50
VOLUME INJ. 2 [ul]	50
SEQUENCE OF INJECTIONS	1->2
DELAY TIME INJ 1/INJ 2 [s]	2.0
MEASURE BACKGROUND	NO
DELAY LAST INJ./MEAS. [s]	0.5
MEASURING TIME [s]	10.0

COMMENT :

SAMPLE		RLU	
1	1	15414887	
	2	2183854	10x
2	3	12688318	
	4	2030952	10x
3	5	3337360	
4	6	4820293	
	7	21285848	
5	8	4139650	
6	9	33804016	
	10	9485125	10x

Grp 1 0.5 mg
Grp 2 5 mg
Grp 5 0 mg

Results indicate substantial knock-down of Luc+ activity by siRNA in liver. Repeat expt with control siRNA to show sequence specificity. Also try injecting pMIR116 & pMIR122 with siRNA against pMIR116 to provide more evidence of specificity.

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
	9/7/01	Recorded by	8/10/01

Project No.

TITLE hp tail vein injection of pMIR116/pMIR122 + s.RNA Book No. 180

From Page No.

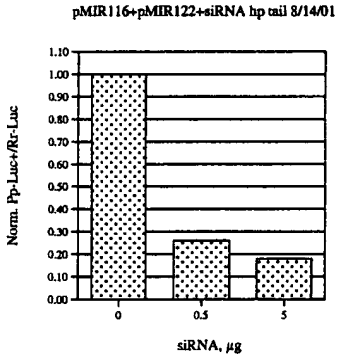
Purpose: Determine if s.RNA is specific to Luc1 in the liver after hp tail vein delivery

Materials:
pMIR116
pMIR122
D257:D258 s.RNA 40 mM 0.53 mg/ml 7/9/01
Promega Dual Luciferase kit

Methods:
Group 1 pMIR116 (10ng) + pMIR122 (1ng) + 0.5 mg s.RNA
Group 2 " + 5 mg s.RNA
Group 3 " w/o s.RNA
Group 4 pMIR116 (10ng)
Group 5 pMIR122 (1ng)

Inject by hp tail vein of ICR mice
Homogenize liver in 2mL lysis Buffer
Dilute 1/100 in 1x PLB
Assay 10µL with Dual Luciferase kit

Results:



Livers looked good at harvest

Inhibition specific to Luc1 target. Rr Luc not affected.

siRNA specific activity occurring in liver.

No effect on Rr Luc suggests interferon pathway not activated in liver by s.RNA

8/14/01
hp tail vein
liver homogenate
Dual Luciferase assay, 10 µl of 1/100 dilution in PLB
10 µg pMIR116/1 µg pMIR122

							group
#6252							
0.5 µg siRNA	1	2	average	total average	Relative #	Normalized	
Luciferase	321860	324268	323064	446307	0.47	0.26	
Renilla	545858	574980	560419	959373			1
#6225							
0.5 µg siRNA	1	2	average				
Luciferase	573550		573550				
Renilla	1358326		1358326				
#6226							
5 µg siRNA	1	2	average	total average	Relative #	Normalized	
Luciferase	332135	349741	340938	220181	0.32	0.18	
Renilla	945021	1031082	988052	691971			2
#6221							
5 µg siRNA	1	2	average				
Luciferase	99424		99424				
Renilla	395891		395891				
#6224							
0 µg siRNA	1	2	average	total average	Relative #	Normalized	
Luciferase	731758	814849	773304	614692	1.78	1.00	
Renilla	457849	507597	482723	345955			3
#6241							
0 µg siRNA	1	2	average				
Luciferase	469566	442595	456081				
Renilla	219192	199180	209186				
#6262							
10 µg pMIR116 only	1	2	average	total average	Relative #	Normalized	
Luciferase	2181219		2181219	1505354	379.37	213.52	
Renilla	4920		4920	3968			4
#6245							
10 µg pMIR116 only	1	2	average				
Luciferase	829489		829489				
Renilla	3016		3016				
#6261							
1µg pMIR122 only	1	2	average	total average	Relative #	Normalized	
Luciferase	12237		12237	6824	0.02	0.01	
Renilla	793405		793405	651220			5
#6264							
1µg pMIR122 only	1	2	average				
Luciferase	1410		1410				
Renilla	509034		509034				

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
	9/7/01	Recorded by	8/14/01

From Page No.

Purpose: Determine if RNAi works in different organs in mice

Materials:

pMiR116	3.79 ug/ul	make 1 ug/ul dilution in Endofree TE	131.9 ul + 368.1 ul TE
pMiR122	2.46 mg/ml	make 0.1 mg/ml dilution	20.3 ul + 479.7 ul TE
DL57:DL58 siRNA	40 uM	0.53 mg/ml ~~duplex 7/9/01~~ see below 7/12/01a	make 0.5 mg/ml dilution
DL64	0.1 nmol/ul	0.67 mg/ml	in RNase free H_2O
DL65	0.1 nmol/ul	0.68 mg/ml	

Methods:

Anneal DL64 and DL65

DL64	100 ul ✓	10 nmol	
DL65	100 ul ✓	10 nmol	⇒ 40 uM duplex
1M Tris pH8	12.5 ul ✓	50 mM final	0.54 mg/ml
5M NaCl	5 ul ✓	100 mM final	
H_2O	32.5 ul ✓		
	250 ul		

Prog #19

94°C	2'
90°C	1'
20°C	1' 1°/min
4°C	∞

make 0.5 mg/ml dilution for ACH

Anneal more DL57 and DL58

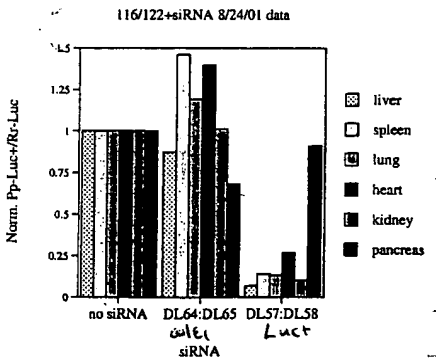
DL57	100 ul	10 nmol ✓	
DL58	100 ul	10 nmol ✓	
1M Tris pH8	12.5 ul	50 mM ✓	
5M NaCl	5 ul	100 mM ✓	
H_2O	32.5 ul	✓	
	250 ul		

Prog #19 ⇒ 40 uM
0.53 mg/ml
Make 0.5 mg/ml dilution for ACH

Group 1 pMiR116(10mg) + pMiR122(1mg) + DL57:DL58 siRNA (5mg)
Group 2 " + DL64:DL65 siRNA (5mg)
Group 3 " no siRNA

Results: 93% 86% 87% 73% 90% 0% mice

See RNAi in liver, spleen, lung, heart, kidney.
In pancreas no RNAi is observed. Degradation by pancreatic RNases? or rather only 8% in tube.
NO RNAi observed with DL64:DL65 directed at plasmid backbone (colE1 ori).



To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
	9/7/01	Recorded by	8/22/01

From Page No.

Purpose: Investigate whether morpholino annealed to DNA oligo bridge allows morpholino uptake in vivo using high pressure tail vein injection.

Materials: see p. 12

- FITC-morpholino control oligo - GeneTools (DL012)
  1,000 pmol/λ 8.3 mg/λ in TE
- Unlabeled DNA oligo DL010
  100 pmol/λ 0.97 mg/λ in TE
- Cy3 labeled DNA oligo DL011
  100 pmol/λ 1.01 mg/λ in TE
- pMIR248 endofree Maxi 2.90 mg/λ in H_2O 9/14/00

Methods:

Anneal oligos in TE (10 mM Tris, 0.1 mM EDTA) + 150 mM NaCl

#		Morpholino	DL010	DL011	TE	5M NaCl	tot vol	mg/λ	Run on gel	need
1	2.5×	12.6λ ✓ 12,600 pmol 104.8 mg	126λ ✓ 12,600 pmol 122 mg	–	~~+4λ~~ Dr. 9/21/00	4.2λ ✓	~140λ	1.62	(3.5×)	8
2	2×	3.6λ ✓ 3,600 pmol 30 mg	–	–	35.2 ✓	1.2λ ✓	40λ	0.75	(1×)	2
3	2×	–	–	3.6λ ✓ 3600 pmol 36.4 mg	2.8λ ✓	1.2λ ✓	40λ	0.91	(1×)	2
4	1×	–	36λ ✓ 3600 pmol 35 mg	–	2.8λ ✓	1.2λ ✓	40λ	0.87	(1×)	1

Anneal 94°C 2' → 80°C → 1°/degree → 10°C → 4°C ∞ (#19)

Ligate 40λ of morpholino:oligo annealed (DL010) ×3
- ✓ 40λ DNA (64.8 mg)
- ✓ 4λ 10× Buffer ligase
- 2λ Ligase (400 u/λ)
- 46λ

Ligate 16°C o/N

Label 60 mg pMIR248 w/ LabelIT Cy3

dH_2O	189.3 ✓
10× A	30λ ✓
pMIR248	20.7λ ✓ (60 mg)
Cy3 LabelIT	60λ
	300λ

Incubate 37°C, 1 hr
Start: 2:15 pm
End: 3:15 pm

Dr 9/27/00
Clean up ~~on 6 columns~~ by EtOH ppt
Wash w/ 70% EtOH
Resuspend 30λ ⇒ 2 mg/λ

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Witnessed & Understood by me,	Date	Invented by	Date
[signature]	10/10/00	Recorded by [signature]	9/21/00

Project No.
Book No. 152 TITLE Injecting morpholino: oligo into M.ce cont'd

From Page No. 55

• Injections to do

#animals		vol	mg	code	tot vol	morpholino mg/animal
• 3 Morpholino: oligo	1	40n	65	DL1	120n ✓	30
3 morpholino: oligo ligated	1-4	40n	65	DL2	120n ✓	30
2 Morpholino	2	40n	30	DL3	80n ✓	30
2 oligo Cy3	3	40n	36.4	DL4	80n ✓	-
pTTR248-Cy3			30		morph ✓ DNA 30n ✓	-
• 2 morpholino: oligo pTTR248-Cy3	1	55n (65+30)	95	DL5	(80n + ~~15n~~) ~~x 2~~ = 160 ~~30n~~ DL 9.27.00	30

morph | oligCy3 | oligo | Morph: oligo | Morph: oligo lig

6el - 3% Agarbel H/S ul EtBr (10n in 80-1 of 1mg/ml sdn)

M	DL3	DL4	#4 (animal)	DL1	DL2	
	2.67	2.20	2.3n	1.23	1.41	vol (2 mg total each)
	6.33	6.8	6.7	7.77	7.59	TNE 10-M Tris 150-M NaCl 0.1 EDTA
	1x →					10x Buffer

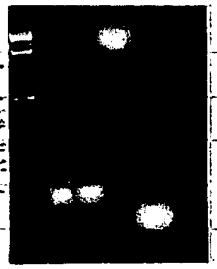
Load - M DL1 DL2 DL3 DL4 4 2hr 60V start: 10:45a

end:

Results:

- Morpholino alone barely migrates into the gel - uncharged
- morpholino: oligo annealed results in all morpholino being take into the gel. Size range ~60 - 200 bp although hard to estimate size of these copolymers as only one of the strands is charged. The fact that the morpholino is taken into the gel indicates that it is paired w/ the oligo. Most is probably annealed to 1 oligo or maybe 2. Longer oligomers are probably much less stable.

Able to see green fluorescence of morpholino and red fluorescence of oligo Cy3 under 302 nm light.



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Witnessed & Understood by	Date	Invented by	Date
	12/22/00	Recorded by	9/21/00

From Page No. 56

Collect livers 24 hrs later
Cut sections (10 μ)
Fix in 1:10 formaldehyde:PBS RT 15'
Wash 3x5' PBS
Incubate in 1:10,000 ToPro-3 20'
Rinse in PBS
Mount

Get 1 slide/animal in each group - will cut others on Mon. if necessary

3	DL1	morph:oligo	
3	DL2	morph:oligo 1 slated	high bkgrd
2	DL3	morph	try MeOH step
2	DL4	oligoCy3	label all as: DL# slide # mouse #
2	DL5	morph:oligo + pMIRCy3	DL1, slide 2 → 1-2-3 / 1-2-3
			DL1, slide 3 → 1-3

~~Confocal~~
Cut remaining slides Mon
Fix these w/ 1:10 formaldehyde as above
wash 1x5' PBS
Place in 100% MeOH 10'
Wash 3xPBS 5'
Incubate in 1:40,000 ToPro-3 20' (1.75 μ in 70 mls)

Injections

Group 1 (DL1) 1 - great inj.
2 - not great
3 - not great
Group 2 (DL2) 1 - great
2 - great
3 - great
Group 3 (DL3) 1 - great
2 - great
Group 4 (DL4) 1 - great
2 - great
Group 5 (DL5) 1 - great

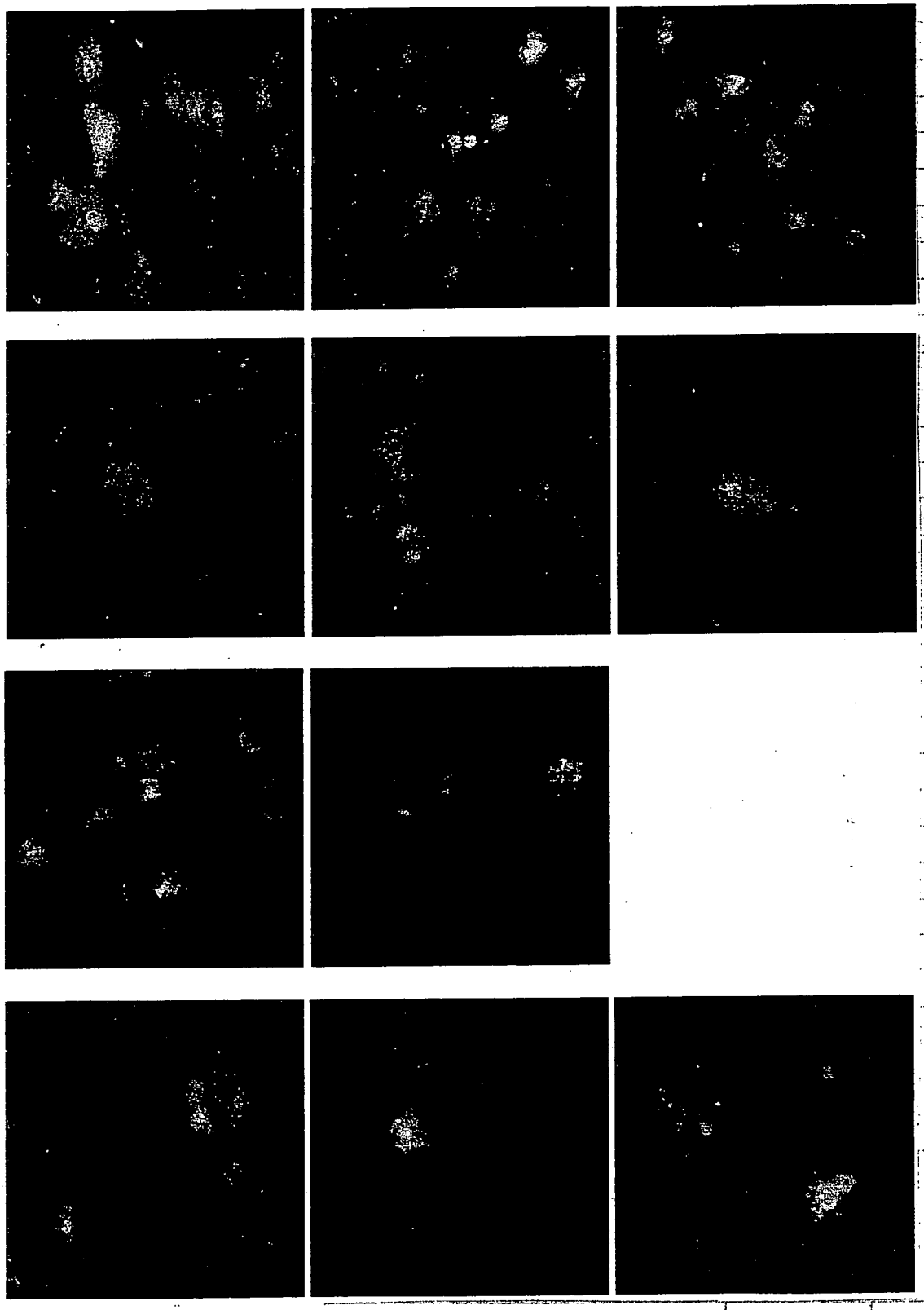
To Page No. 70

Witnessed & Understood by me	Date	Witnessed by	Date
Jennifer	10/10/00		9/24/00

Project No.
Book No. 152 TITLE Injecting morpholino : oligo into mice cont'd

From Page No. 69

Results:



No. 71

Witnessed & Understood by
Date 10/10/00

Invented by
Recorded by
Date 10/2/00

Title: Injecting morpholino oligo into mice cont'd

From Page No. 70

Conclusion:

1. Morpholino alone able to enter cells! Pattern is diffuse cytoplasmic + nuclear staining. This is unexpected. Route of uptake?
2. Morpholino:oligo able to enter cells as well. Seem to be fewer cells but with morpholino alone, but small sample size make conclusions difficult. No difference if DNA strand is treated with ligase.
3. When morpholino:oligo + Cy3 labeled DNA (plasmid) are injected simultaneously, both enter same cells and to similar relative amounts. Plasmid DNA can be seen as punctate nuclear staining in some cells, punctate cytoplasmic + nuclear staining in other cells, and cytoplasmic only, in a majority of cells. In some nuclei w/ plasmid DNA + morpholigo, both seem to be located in same sub-nuclear locations as punctate staining.

Investigate:

1. Time course of uptake
2. Inhibition of gene expression
3. low pressure injections

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date
[signature]	10/10/00	Recorded by [signature]	10/2/00